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Microbiology: lessons from a first attempt at Lake Ellsworth

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During the attempt to directly access, measure and sample Subglacial Lake Ellsworth in 2012–2013, we conducted microbiological analyses of the drilling equipment, scientific instrumentation, field camp and natural surroundings. From these studies, a number of lessons can be learned about the cleanliness of deep Antarctic subglacial lake access leading to, in particular, knowledge of the limitations of some of the most basic relevant microbiological principles. Here, we focus on five of the core challenges faced and describe how cleanliness and sterilization were implemented in the field. In the light of our field experiences, we consider how effective these actions were, and what can be learnt for future subglacial exploration missions. The five areas covered are: (i) field camp environment and activities, (ii) the engineering processes surrounding the hot water drilling, (iii) sample handling, including recovery, stability and preservation, (iv) clean access methodologies and removal of sample material, and

(v) the biodiversity and distribution of bacteria around the Antarctic. Comparisons are made between the microbiology of the Lake Ellsworth field site and other Antarctic systems, including the lakes on Signy Island, and on the Antarctic Peninsula at Lake Hodgson. Ongoing research to better define and characterize the behaviour of natural and introduced microbial populations in response to deep-ice drilling is also discussed. We recommend that future access programmes: (i) assess each specific local environment in enhanced detail due to the potential for local contamination, (ii) consider the sterility of the access in more detail, specifically focusing on single cell colonization and the introduction of new species through contamination of pre-existing microbial communities, (iii) consider experimental bias in methodological approaches, (iv) undertake *in situ* biodiversity detection to mitigate risk of non-sample return and post-sample contamination, and (v) address the critical question of how important these microbes are in the functioning of Antarctic ecosystems.

1. Introduction

Since the discovery of Antarctic subglacial aquatic ecosystems, there has been a rapid expansion in our understanding of these environments, their number [1], diversity [2] and interconnectivity [3]. More importantly, technological and logistical capabilities have made deep Antarctic subglacial lake access both feasible and scientifically compelling in recent years [4].

One of the key goals of Antarctic subglacial lake exploration has been to measure, analyse and understand life in these extreme environments [5]. Microbiological activity and function have already been shown to be present in such systems, albeit at the ice sheet margin [6]. This might be expected, as microorganisms are known to withstand the range of selection pressures and indeed have been predicted to exist within deep subglacial environments [7]. Indeed, microbes have been shown to exist in analogous extreme environments, such as deep sea sediments [8], to display a huge diversity, to have the ability to exist in the absence of photoautotrophy and to remain dormant in isolation for hundreds of thousands to millions of years.

The ecosystem types targeted for exploration comprise a diversity of habitat types, including those that are ice covered (Lake Vida), emerging from under the Antarctic ice sheet (Lake Hodgson), sediments (Kamb ice stream), the largest (Lake Vostok), potentially most microbiologically diverse (Lake Whillans) and close to the ice divide (Lake Ellsworth). What is likely, from the information emerging to date, is that Antarctic subglacial lakes follow the patterns of Antarctic supraglacial lakes, in that while they may turn out to have features in common, every system might come to be regarded as unique.

To advance knowledge of subglacial lake microbiology, three ambitious projects aimed to access discrete subglacial aquatic systems and retrieve samples for analysis. These projects built on earlier observations of microbial activity made at the Kamb Ice Stream [9], Lake Vostok accretion ice [9,10], Blood Falls [11], Lake Vida [12] and Lake Hodgson [13]. In this paper, attention is focused on one of these access targets—Subglacial Lake Ellsworth [14,15].

Subglacial Lake Ellsworth lies at the bottom of a deep trough, 3 km beneath the ice surface at the centre of the West Antarctic Ice Sheet about 70 km west of the Ellsworth Mountains. Seismic studies reveal that this long narrow lake is approximately 150 m deep and covers an area of 29 km^2 [14,16]. In 2012, an experiment was conducted to explore this lake using a purpose built clean deep-ice hot water drill [14].

Lake Ellsworth, as all other Antarctic environments, is not only located in the most remote continent on the Earth, but has been physically separated from other continents since the detachment of Antarctica from the Gondwana supercontinent approximately 100 Ma ago. This region is isolated from the rest of the world by the Southern Ocean, the Antarctic circumpolar current and the circumpolar vortex. The configuration of the Southern Ocean, in fact, presents a major barrier to potential colonizers of Antarctica by introduced species [17] and the absence of industry and relatively low human impact have often earmarked the Antarctic as a region where we can study the global effects of human activity. Nonetheless, diverse microbial communities

Table 1. Some of the key bacterial groups identified in Antarctic snow and ice. The table shows clear differences across adjacent substrates.

snow		ice cores		subglacial	
Russian stations	Lopatina *et al.* [18]	Mizuho base, Yamato mtns	Segawa *et al.* [19]	Kamb Ice Stream	Lanoil *et al.* [20]
Variovorax sp.		Firmicutes		Betaproteobacteria	
Janthinobacterium sp.		Gammaproteobacteria		Alphaproteobacteria	
Pseudomonas sp.				Actinobacteria	
Sphingomonas sp.		Vostok accretion ice	Shtarkman *et al.* [21]		
			Rogers *et al.* [22]	Blood Falls	Mikucki & Priscu [23]
Halley		Firmicutes			
		Proteobacteria		*Thiomicrospira arctica*	
Dome C	Van Houdt *et al.* [24]	Actinobacteria		Betaproteobacteria	
				Deltaproteobacteria	
Paenibacillus sp.		coastal ice core	Antony *et al.* [25]	Gammaproteobacteria	
				Bacteroidetes	
coastal inland transect	Antony *et al.* [25]	*Methylobacterium* sp.			
		Brevundimonas sp.		Vostok drill fluid	Alekhina *et al.* [26]
Cellulosimicrobium sp.		*Paenibacillus* sp.			
Bacillus sp.		*Bacillus* sp.		*Sphingomonas* sp.	
Ralstonia sp.		*Micrococcus* sp.			
				Vostok accretion ice	Christner *et al.* [27]

(*Continued.*)

Table 1. (*Continued*.)

snow		ice cores		subglacial	
Langhovde	Fujii *et al.* [28]	Collins Glacier	Garcia-Echauri *et al.* [29]	*Brachybacteria* sp.	
Hymenobacter sp.		Betaproteobacteria		*Methylobacterium* sp.	
		Gammaproteobacteria		*Paenibacillus* sp.	
Dumont d'Urville	Gonzalez-Toril *et al.* [30]	Alphaprotobacteria		*Sphingomonas* sp.	
		Gram-positive high GC			
Limnobacter sp.		CFB		Subglacial ice above Vostok	Priscu *et al.* [31]
Pseudonocardia sp.		Gram-positive low GC			
Alphaproteobacter				Alphaproteobacteria	
Brevundimonas sp.		Victoria Upper Glacier	Klassen & Fought [32]	Betaproteobacteria	
				Actinomycetes	
Carpenter *et al.* [33]	South Pole snow	*Hymenobacter* sp.			
Deinococcus sp.					

Table 2. Fundamental questions in subglacial microbiology. Some of the fundamental questions in microbiology that still remain and need to be properly addressed in developing deep subglacial lake access technology.

How many cells are needed in an inoculum to establish a viable colony?
What ecological role do dormant cells have, indeed, are they only relevant if they are dormant?
Do introduced cells interact through signalling with the indigenous population, for example, through quorum sensing or quorum quenching?
Are deep subglacial lake microbial ecosystems stable or dynamic?
In terms of establishment, how dominant are introduced cells which are unlikely to be highly adapted to the subglacial lake selection pressures, when compared to the existing, established dominant communities?
Are introduced cells competitive, e.g. can they form biofilms?
Does the particle association of microorganisms affect collection strategies?
Are microbial growth rates viable in a potentially unfavourable environment?
What are the effects of a potentially huge induced selection pressure on introduced species?
How plastic are the introduced microorganisms genetically, can they adapt to subglacial conditions, e.g. low temperature adaptations, pressure, no light?
Within deep Antarctic subglacial lakes how important is the rare diversity, how much functional redundancy exists and is there a core environmental genome?
Is existing population resilience more likely as they are better adapted than potential colonists (cf. human pathogen emergence from these environments is unlikely as there are no suitable 'hosts')?
Are Antarctic indigenous microbial communities highly competitive after all?

have been reported in the snow and ice from the Antarctic (table 1; see also table 2 in Pearce [34]), following similar studies in other regions in the cryosphere, including snow from the Alps [35], the Tibetan plateau [36], Arctic Svalbard [37,38], Arctic Greenland [39], Arctic Alaska [40] and Antarctic snow (this study, [41]). Microbial communities differ significantly across the environments of nthe cryosphere [42], and this also applies to Antarctica, including both supraglacial lakes [43] and subglacial lakes, with Ellsworth potentially harbouring unique microbial communities.

Set against this background, the presence of different communities in different environments makes the transmission of microorganisms from snow, into the melt tank for drilling fluid, then through the ice and into the subglacial lake, potentially very important as it represents moving new microbes into an environment where they had not existed before. The risk of doing this is still unknown.

In addition, technological limitations to fully understand microbial ecology in extreme polar environments still exist. Of particular concern, is that when microbiological investigation is technically challenging, there is a tendency to accept the limitations, thus restricting scientific outcomes, or simply to overlook them, which also limits scientific discovery. Technological advances over the last few years mean that it is now possible to understand these limitations and to mitigate their influence on the science. Fundamental questions in microbiology remain and still need to be addressed (table 2), which make the use of novel technological applications compelling.

One such technological limitation relates to the issue of sterilization. This is a term used to refer to any process that eliminates or kills all forms of microbial life. Hence, sterility ought to refer to the absence of life. However, our definitions of sterility for practical purposes, and particularly for its assessment, rarely reach this ideal. Many of the planetary protection criteria (http://planetaryprotection.nasa.gov/methods) talk of bioburden reduction and are constrained by the need for research and technology development, which in some cases are required to enable ambitious future planetary exploration missions [44]. In the pharmaceutical industry (http://www.fda.gov/downloads/Drugs/. . . /Guidances/ucm070342.pdf), on the other hand,

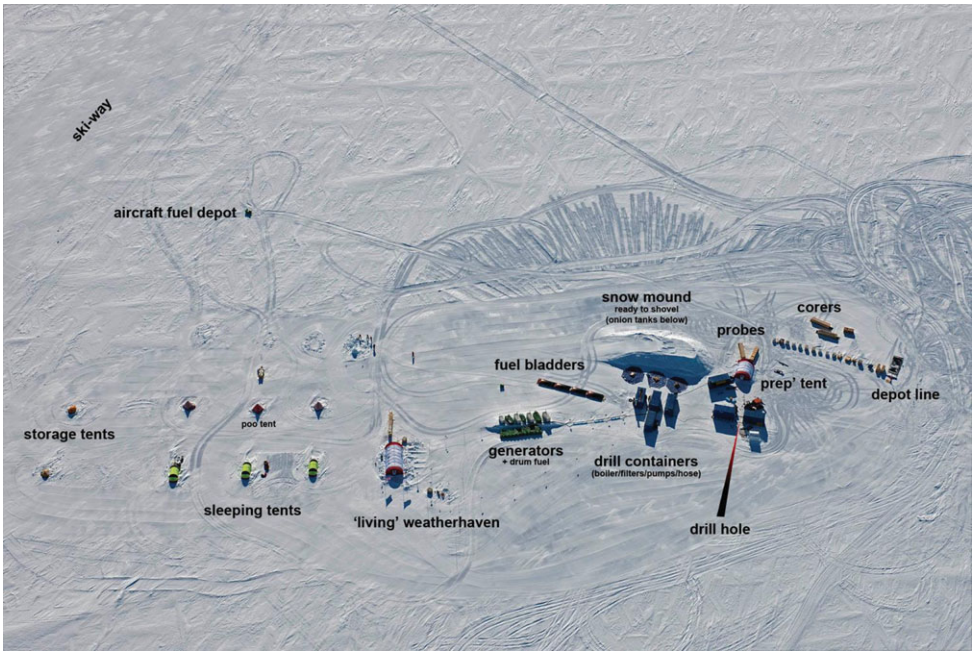

Figure 1. Lake Ellsworth field camp. Site map showing the locations of the drill site ('drill hole' on image), generator site ('generators' on image) and kitchen ('living weatherhaven' on image). Scale: drill containers are standard shipping containers. (Online version in colour.)

sterility tests are limited in their ability to detect contamination because of the small sample size typically used. Green [45] in stating that the field of surgical sterilization and surgical safety is less confused by technical inconsistencies identified the need to learn how to translate sterility tests in terms of real world infection hazards. For regulatory purposes, in the food industry, it has been quoted that 'absolute sterility can never be reached or guaranteed'.

The general principle used in the Lake Ellsworth programme was to target 'no measurable microbial populations' to be present on any engineered structures in contact or communication (i.e. the probe, tether, sediment corer and thermistor string if deployed) with the subaquatic environment [14]. Indeed, all engineered structures were checked both after manufacture and once ready for shipping, to determine the presence of microbial populations (i.e. to confirm that the principle was being upheld). Components of the probes and corers were assembled in a clean room environment to ISO 14644 (cleanliness for equipment used in clean rooms) working to Class 100 000 (ISO 8). Once assembled, the instruments were transported to Antarctica in sealed bags, to be opened only once in the sterilized environment of the borehole.

The issue of potential contamination is also scalable to the size of the Antarctic field camp required for the lake access operation (figure 1), and with logistic movements and personnel accessing the site, as it is inevitable that some human impact will be felt in the local environment. As part of the Lake Ellsworth programme, a number of advances were made in how to approach and deal with microbial contamination issues [14]. These included assessment of sterility techniques, manufacturing of the probes to high levels of cleanliness, designing the hot-water drill to be clean and testing of sterility techniques during field operations. A comprehensive environmental evaluation [14], discussed at the 2011 and 2012 Committee on Environmental Protection, at the Antarctic Treaty Committee Meetings, identified three potential areas of concern regarding contamination of the field site: (i) material brought into the Antarctic from the UK or any of the tropical and temperate locations passed in transit, (ii) material taken from the surface snow used to generate drill fluid which might enter the lake, and (iii) material melted out of the

ice itself which was not yet in the subglacial ecosystem. The latter may introduce microbes into the lake that might otherwise have perished due to longer residence times in the ice matrix.

The Lake Ellsworth programme demonstrated how a high degree of environmental stewardship can be assured in deep-field Antarctic exploration programmes. However, such assurance should not be restricted to projects that aspire to search for life. While they are not generally assumed necessary for geological or glaciological projects, their use in such work may be just as necessary to avoid contamination of pristine environments. Despite development in cleanliness protocols, when investigating environmental samples in remote locations, using hot water drills and probes, particularly when human activity is involved, it is impossible to guarantee sterility. Uncertainty can therefore remain around the quality and integrity of both the samples obtained and the long-term effects on the subglacial system under investigation. In consequence, it is important that the costs involved in the development and use of drill and probe systems that ensure clean access and sampling are fully incorporated into all subglacial access programmes.

So, the key technical challenges in sterility and cleanliness relate to (i) the ability to generate 'sterile' materials, (ii) the ability to demonstrate that they are indeed sterile, and (iii) knowing the environmental impact if they are not sterile. Most sterility testing is still based upon the number of colony forming units per unit volume or surface area. However, the VBNC (or Viable but Non-Culturable) state has been known for some time [46]. Indeed only a relatively small (but variable) proportion of cells can be grown in culture. The impact of introducing cells that have not been detected in culture but are still viable is yet to be determined.

Here, we present the results of a combined laboratory and field study at the Lake Ellsworth drill site (Nov 2012–Jan 2013) to identify means to mitigate the effects of human impact in the lake through technology and logistics operations. Specifically, three key sites were sampled in an attempt to establish whether human impact was detectable in the environment as follows: (i) outside the mess-tent kitchen, due to the level of human traffic and the presence of non-indigenous microorganisms in the food being handled there, (ii) around the fuel blubbers, due to the potential for hydrocarbon spills and the handling and transfer of potential microorganisms that the fuel could contain, and (iii) in the waste management area.

2. Material and methods

(a) Sterility validation of engineering materials

During the manufacture and assembly of the lake measurement probes [47] (figure 2), swab samples were collected in order to assess the efficiency of the four-stage sterilization process undertaken (detergent wash, ethanol rinse, a commercially available biocide (Tenon Biocleanse) and hydrogen peroxide vapour exposure) as previously reported [48]. Randomly chosen titanium grade V surfaces of the probes were sampled with human DNA-free SK-1S DNA Buccal Swabs (Isohelix), which were wetted with PCR grade water (Sigma) in a sterile environment. Surfaces were sampled in a unidirectional horizontal manner holding the swab at an approximately 30° angle to the surface for 30 s for each side of the swab. Swab heads were aseptically placed in a 5 ml tube and sealed until further analysis. A total area of 314 to 528 cm^2 was sampled per swab. Swabs wetted with PCR grade water were used as blanks. To remove the sampled material from the swabs 1 ml of PCR grade water (Sigma) was added to the tubes followed by vortexing for 30 s and 5000*g* centrifugation for 5 min. The liquid sample was transferred into sterile 1.5 ml centrifuge tubes for DNA extraction. DNA was extracted using the DNeasy Blood and Tissue DNA Extraction kit (Qiagen). Total dsDNA concentration was measured based on fluorescence after staining the DNA extracted from the swabs using the Quant-it PicoGreen kit (Invitrogen). For the detection of bacteria, the extracted DNA was amplified using Universal Bacterial Primers 27F (5′-AGAGTTTGATCCTGGCTCAG-3′) and 1492R (5′-GNTACCTTGTTACGACTT-3′) at a final concentration of 10 µM. Taq DNA polymerase (NEB) and Standard Taq buffer (NEB) were used. DNA was initially heated up to 94°C for 2 min followed by 60 cycles of 94°C (1 min),

Figure 2. Probe handling in field and laboratory conditions. Construction of the probe in a specially constructed containment facility. (Online version in colour.)

50°C (1 min) and 68°C (1.5 min) and a final elongation step of 68°C for 10 min. PCR products were initially analysed using gel electrophoresis while for increased sensitivity an Agilent 2100 Bioanalyser with the High-Sensitivity DNA kit (Agilent) was used. Water sample containers and filters were visibly checked for sterility and for behaviour following forward contamination with bacterial cells using scanning electron microscopy.

(b) Microbes in the field site surface snow

Bacteria from the surface snow (10 l) were collected by filtration onto 0.2 µm pore-size polycarbonate filters. Genomic DNA was extracted directly from the filters using the MoBio PowerWater DNA isolation kit (MoBio, CA, USA) according to the manufacturer's instructions. The V4 region of the 16S rRNA gene was amplified in 25 µl PCR reactions containing 1 µM primers, 0.2 mM dNTPs (NEB) and 1.25U of Taq Polymerase (5Prime, HotMaster Taq) with the recommended PCR buffer. The primers used for the amplification of the 16S rRNA gene were the universal 515F (5′-GTGCCAGCMGCCGCGGTAA-3′) and 806R (5′-GGACTACHVGGGTWTCTAAT-3′) as suggested by Caporaso *et al.* [49] containing the appropriate adaptors. Samples were amplified using an initial denaturation step of 2 min at 94°C, followed by 15 cycles of 45 s of denaturation at 94°C, 60 s annealing at 50°C and 90 s elongation at 70°C followed by a final elongation step at 70°C for 10 min. The amplicons were used as template for a 15 cycles nested PCR with the same conditions and reagent concentration to increase the PCR efficiency. Amplicons were sequenced on a MiSeq Illumina Sequencer (Illumina) in Liverpool (Centre for Genomic Research, University of Liverpool, UK). Raw output files were trimmed with Cutadapt (v. 1.2.1) followed by a further trimming with Sicle (v. 1.200) with a minimum quality score of 20. Both pair reads with a minimum length of 290 bp were uploaded to the BaseSpace platform (Illumina) for assembling. The assembled reads were taxonomically classified against

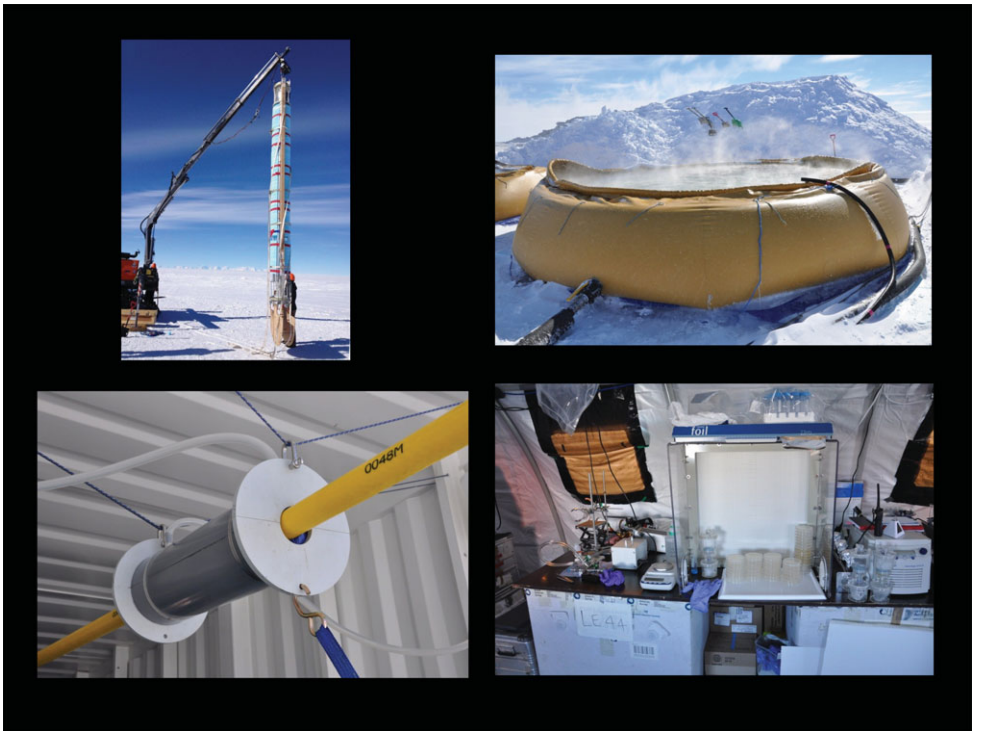

Figure 3. Probe handling in field and laboratory conditions. Field environments where samples would be handled. (Online version in colour.)

the GreenGenes taxonomic database (version May 2013) using the 16S Metagenomics application (v. 1.0.1). Diversity indexes were estimated and principal components analysis (PCA) graphs were generated with Past software (v. 3.0).

(c) On site handling of engineering and maintenance of sterility

During Lake Ellsworth field operations, prior to hot-water drilling, the probe and corer were set up and moved into position in a dry practice run of the deployment procedure (figure 3). The container which housed the probe was lifted into place above the drill hole and was connected via an 'air-lock' to a sterile dummy 'well-head'. Throughout the process, the equipment was swabbed and checked for contamination (figure 4). On return to the UK, the boxes were opened within a clean containment facility and the probes were again swabbed for contamination.

(d) Limits of detection

In order to estimate the limit of detection of the DNA extraction and amplification method that we applied, an overnight *Pseudomonas fluorescens* (NCTC 10038, Health Protection Agency, UK) culture was prepared with the *Pseudomonas*-specific King's B medium (20 $g l^{-1}$ peptone, 6.6 mM potassium phosphate dibasic trihydrate, 6.1 mM magnesium sulfate heptahydrate and 1.4 M glycerol) [50] at 25°C and continuously agitated in an orbital-shaking incubator (Stuart Equipment, UK) at 200 r.p.m. Starting with a *P. fluorescens* culture concentration of 7.74×10^7 cells ml^{-1}, the culture was 10-fold diluted. DNA was extracted from 1 ml of each culture dilution (in triplicate samples) and amplified with the same method as with the swab and blank samples. PCR grade water was used for the DNA extraction blank samples (1 ml) and PCR blank samples. In total, two replicate experiments were performed.

Figure 4. Probe handling in field and laboratory conditions. Attachment of the probe to the tether inside a portable glove box in the field. (Online version in colour.)

(e) Potential changes to sample integrity

A variety of changes are possible during sample recovery from the lake to the ice surface. The floor of Lake Ellsworth is 3.2 km beneath the surface of the ice. As a result the probe would have taken some time (several hours) to acquire and recover the samples. With a significant drop in pressure on recovery from the lake to the surface (i.e. from at least 300 bar down to atmospheric pressure, exceeding this considerably in samples frozen within their chambers) and potential freeze/thaw temperature cycles on sample recovery, the possibility existed of significant induced physiological change in the sample.

To test the effect of hydrostatic pressure change on Antarctic surface microbiota, bacteria isolated from the surface snow at the Ellsworth site were first identified and analysed. Droplets of melt water from the surface snow were placed on R2A agar (Sigma) plates [51] and incubated at 4°C until colony formation. Colonies of different colour and characteristics were isolated. Genomic DNA was extracted using the DNeasy Blood and Tissue DNA Extraction kit (Qiagen) and the 16S rRNA gene was amplified using Universal Bacterial Primers 27F and 1492R as described above. To identify the isolates the resulting amplicons were individually sequenced (Eurofins Genomics) and the 16S rRNA gene reads were then subjected to BLAST sequence similarity search [52] to identify the nearest taxa. An overnight culture of isolated bacteria closely related to *Hymenobacter* sp. was prepared in R2A liquid medium using the same recipe but excluding the agar (0.5 gl^{-1} yeast extract, 0.5 gl^{-1} proteose peptone, 0.5 gl^{-1} casein hydrolysate, 0.5 gl^{-1} glucose, 0.5 gl^{-1} soluble starch, 0.3 gl^{-1} dipotassium phosphate, 0.3 gl^{-1} sodium pyruvate and 0.024 gl^{-1} magnesium sulfate) incubated at 25°C with continuous agitation at 200 r.p.m. The culture was centrifuged at 5000*g* for 2 min and suspended in sterile phosphate-buffered saline. Cultures were placed in sterile 150 ml Flexboy Bags (Sartorius) prior to pressure tests. The culture was pressurized to 300 bar to simulate the *in situ* pressure of subglacial environments [53] in the pressure test tank PV2 of the NOCS Pressure Testing Facility. A fast (10 min pressurization followed by 2 s depressurization) and a slow (2 h pressurization and 2 h depressurization) cycle were used to assess the effects of pressure change rate. Samples kept at atmospheric pressure were used as controls. Cell viability was assessed with the standard plate assay. Cell abundance was calculated in samples stained with SYBR Green I (Molecular Probes) (final dilution: 4×10^{-4} of the commercial solution) and incubated in the dark at room temperature for at least 15 min using an Apogee A50 Micro Flow Cytometer (Apogee). Scattered light detected by the Forward (FSC) and 90° Side (SSC) detectors was also measured to validate the physical changes in cell membrane, shape and size.

3. Results

(a) Sterility verification of engineering materials

In all the samples, total dsDNA fluorescence was below the quantification limit of the Quant-it Picogreen kit (150 pg). No amplification was detected after the analysis of all the swab and blank samples (figure 5). Water sample containers and filters (figure 6) were visibly checked for sterility and for behaviour following forward contamination with bacterial cells.

(b) Microbes in the site snow

In total, 3 112 567 reads were obtained and passed the quality checks with 69% of the reads classified up to genus level. Some 738 056 reads classified belonged to the three samples collected from the camp sites (Kitchen, Drill and Generator sites) while 1 399 225 belonged to the five samples collected from the surface snow at a 500–1500 m distance from the camp. PCA revealed three and four district groups of samples on the taxonomic levels of phylum, class and order, genus, respectively (figure 7). Samples collected from the kitchen and the generator sites are characterized by the lowest diversity at all taxonomic levels with Shannon species diversity index values of 1.036 and 0.836, respectively, and approximately 735 species identified, whereas the Lake Ellsworth drill site presented the highest Shannon diversity index value among all samples analysed (1.680) with 1487 species identified. In comparison, the samples from the surface snow presented mean Shannon diversity index value of 1.29 ± 0.29 with 997 ± 94 species identified per sample. Alphaproteobacteria are the predominant class group in the Kitchen (56.3%) location consisting of 505 individual OTU (figure 8). Most Alphaproteobacteria sequences (54.4% of all reads) belonged to the *Bradyrhizobium* genus. Alphaproteobacteria were also the dominant class in the Generator site (40.3%), whereas Actinobacteria (30.5%), Alphaproteobacteria (24.1%) and Bacilli (18.3%) were the most common classes at the Drill site. Relative abundances of the 15 main species classified at each Camp sample are presented in table 3.

(c) On site handling of engineering and maintenance of sterility

Subsequent analyses of the swabs revealed that at no stage during any of the handling procedures were contaminants detected on any of the probe surfaces.

(d) Limits of detection

DNA amplification was detected up to the 7th dilution, which corresponded to 7.7 cells (figure 9*a*,*b*). As no amplification from bacterial DNA was detected from the swabs studied to date, we estimate that the bacterial numbers were less than 10 per 300–500 cm^2.

(e) Potential changes to sample integrity

To examine the effects of hydrostatic pressure on bacteria inhabiting the Antarctic snow above Lake Ellsworth two pressure change rates (slow and fast) were tested. Our preliminary data (figure 10) show a significant loss in cell viability of approximately 25% at both pressure change rates (24.6% and 27.6% in fast and slow treatments respectively) as determined by the ability of cells to form colonies. Cell abundance measured by flow cytometry following positive staining with SYBR Green I also presented significant decrease at both pressure change rates (15.7% and 35.1% in fast and slow treatments, respectively). In both cell viability and abundance, no statistically significant changes between the slow and fast pressure changes were detected.

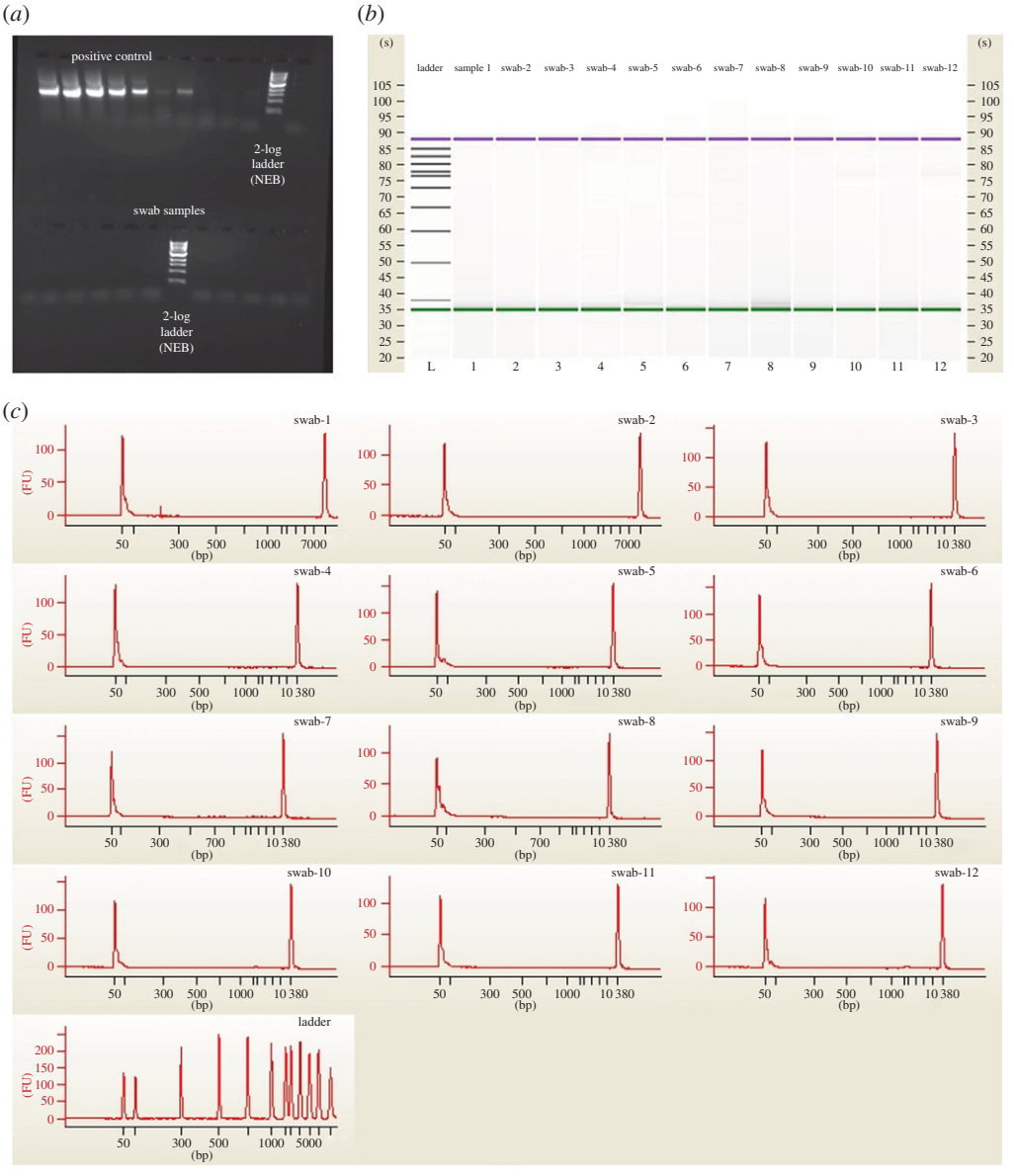

Figure 5. Probe swabbing experiment. (*a*) Gel image showing absence of amplification from any of the test samples (and decreasing concentration from the positive controls where known quantities of cells were added), (*b*) a diagrammatic representation of the gel photograph, showing no detection of microbes from any of the test swabs and (*c*) an electrograph of the swab samples and the ladder that was used based on the High-Sensitivity DNA kit (Agilent Technologies). (Online version in colour.)

4. Discussion

(a) Sterilization of engineering materials

The aim of the Lake Ellsworth programme was to target 'no measurable microbial populations' on any engineered structures [14]. To this end, our results showed that it is possible to demonstrate the sterility of engineering materials to below the limit of detection of contamination and to maintain this apparent sterility throughout deployment, handling and return to the UK.

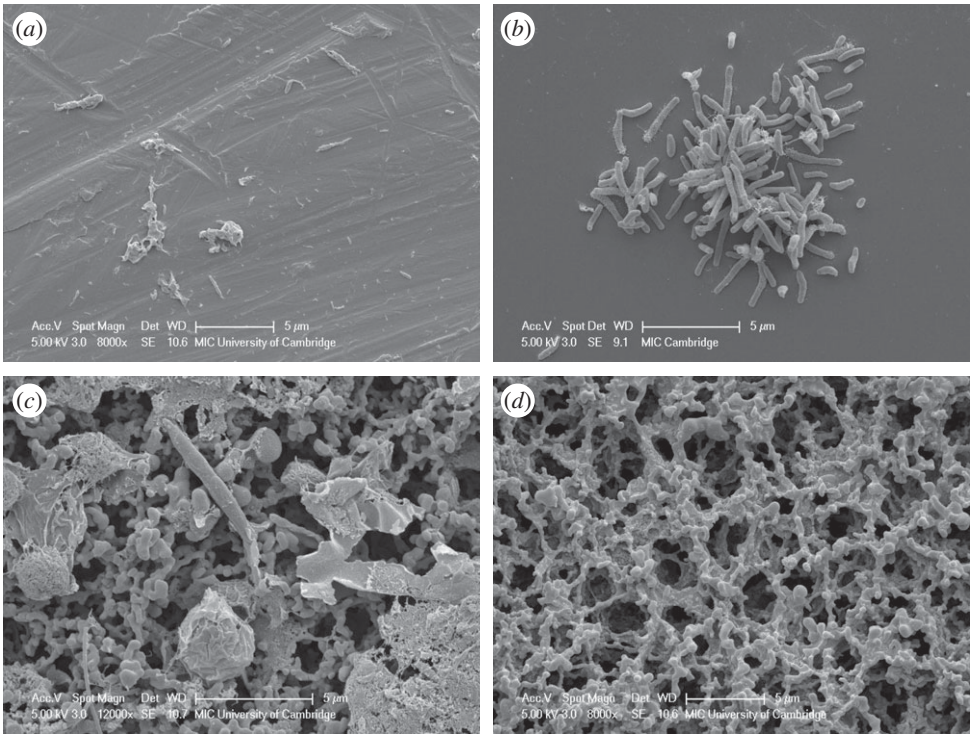

Figure 6. Scanning electron micrographs of a titanium coupon from which the sample bottles were made. (*a*) Post-sterilization, (*b*) forward contaminated, and the filter membranes in the filter housing (*c*) post- and (*d*) pre-sampling.

(b) Microbes in the site snow

Our results suggest that the field camp may have a detectable impact on the bacterial community on the ice. In total, 378 635 reads were classified up to species level from the three Camp samples with 16.1% of them being 97% similar to the *Bradyrhizobium cytisi* sequence; its dominance varied significantly between samples. Sequences classified to *Bradyrhizodium* sp. have been reported from Antarctica such as in the accretion ice above Lake Vostok [54] where they are hypothesized to play a significant role in nitrogen fixation and nitrification and also was reported as one of the most abundant genera in soil community of Mars Oasis on Alexander Island [34]. *Bacillus nealsonii*, a spore forming Gram-positive bacterium, was one of the top species found in all of the Camp samples and, in particular, in the Drill site accounting for 1.5% of all reads up to species level; this species has been previously isolated from a NASA spacecraft-assembly facility [55]. The spores of this bacterium exhibited increased survivability to UV and hydrogen peroxide which were the on-site sterilization methods of choice during the drilling [14]. *Arthrobacter psychrochitiniphilus*, *Janthinobacterium lividum* and *Cellulomonas xylanilytica* were also found to be common between all three samples. Most of the species have been previously identified in environmental samples (e.g. [56,57]). *Propionibacterium acnes* (3.3% of all species reads at the Kitchen site and 3.0% at the Generator site), *Streptococcus pseudopneumoniae* (0.88% at the Kitchen and 0.53% at the Generator site), *Streptococcus oralis* (0.37% at the Kitchen site) and *Staphylococcus haemolyticus* (0.14% at the Kitchen site) were the main potential human-related microbiota found. Bacterial species diversity decreased in the Kitchen and Generator sites due to increased dominance of the nitrogen fixing genus *Bradyrhizobium*. Potentially human-related microbiota were found at the Kitchen site, which demonstrates that further effort may have to be taken in preventing contamination from the camp. Finally, the significantly different bacterial community composition at the drilling site and particularly the presence

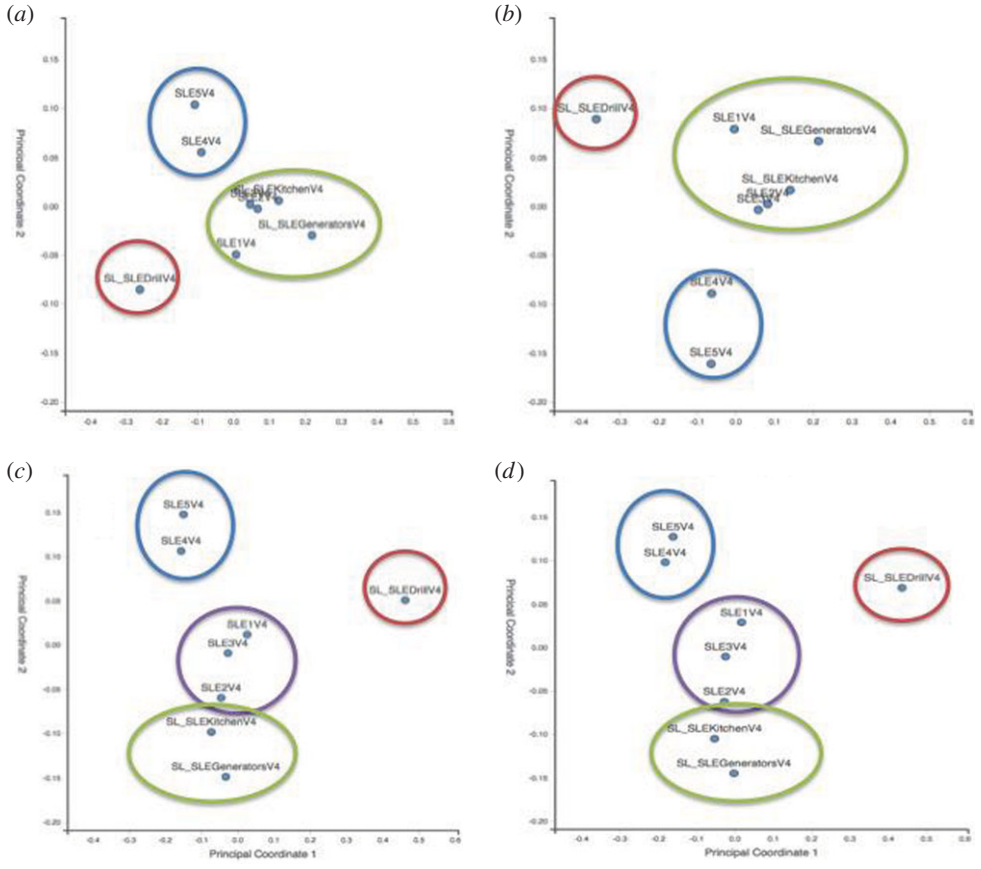

Figure 7. PCA on the different samples analysed. The different groups of samples are marked with coloured circles. The samples collected from the Drill site (red circle) and SLE5 and SLE4 sites (blue circle) that were collected from locations closer to Ellsworth Mountains appeared very different compared with the rest of the samples at all taxonomic levels. The samples from the rest of the snow sites and the Generator and Kitchen sites were initially grouped together up to order level (green circle) while at lower taxonomic levels Camp and snow samples can be grouped separately (green and purple circles).

of spore-forming bacteria resistant to common sterilization methods indicated the potential danger of contaminating the site and then the subglacial lake (via drill fluid) during the drilling process and points to the need to assess the local environment when conducting drilling projects.

(c) On site handling of engineering and maintenance of sterility

As a result of a suboptimal operation of the hot-water drill boiler during field operations [15], soot particles clogged the microbiological filters, which as a consequence needed to be replaced while drilling the initial borehole. This left the field party with only the backup filters to clean the drill water prior to lake entry (figure 11). As a consequence, we retained the 0.2 µm filters until the end of the drilling process, saving them for the moments just prior to lake access. In future, the issue of drill water filtration needs to be looked at carefully, with additional backup and potentially more rigorous pre-filters. In any case, a potentially serious issue with sub 0.2 µm cell filtrate has recently emerged after Luef *et al.* [58] recently suggested that small cells of less than 0.2 µm might be enriched by filtration.

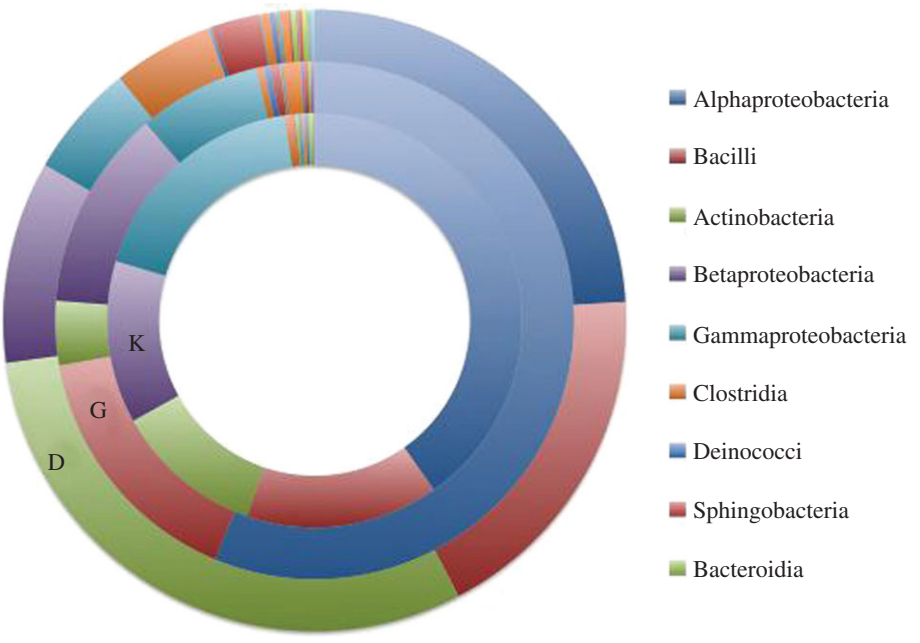

Figure 8. Class level distributions of sequences. Distribution of sequences, at the class level, observed in the Drilling (D), Generator (G) and Kitchen (K) sites.

(d) Limits of detection

A key issue in environmental protection is in providing evidence for the potential of significant harm. This is incredibly hard to demonstrate for work involving remote subglacial lake access. One of the key challenges identified during both pre-season preparation and fieldwork was the limited ability to detect if things had gone wrong in terms of contaminating the lake. To this end, we made a simple contamination calculation for Lake Ellsworth, based upon the detection limits of cells in the assays used, the volume of drill fluid likely to penetrate the lake and the volume of the lake. The detection limit of the various assays available to us was approximately 2–8 cells ml^{-1}. In the sample probe, the total sample volume across all bottles was 1.2 l. Therefore, in order to detect contaminants, we would have needed 9600 contaminant cells to be in the samples. With a total lake volume of 6.4×10^{13} l and assuming any water entering the lake would have been rapidly well mixed, we would need 6.4×10^{17} cells of contamination in the lake in order to detect them. It is extremely unlikely, therefore, that any cells entering the lake within the drill fluid would ever be detected in the lake itself. We believe such calculations are of value for other subglacial lake investigations, to comprehend whether potential contamination can ever be detectable.

All methodologies in microbiology have some form of bias associated with them. In this study, further methodological considerations included the following. (i) The DNA extraction methodology: Vishnivetskaya *et al.* [59] demonstrated that genomic DNA yields differ between the extraction kits, but reproducible bacterial community structure analysis may be accomplished using genomic DNAs from the three bead-beating lysis extraction kits for permafrost samples. (ii) The lack of 100% coverage of diversity, inherent in almost all molecular methodologies could exclude important taxa—many rare-biosphere taxa have important ecological roles, as described below, serving as nearly limitless reservoirs of genetic and functional diversity [60]. (iii) Lake samples determined in the same system at the same time on subsequent seasons can yield completely different results (figure 12). (iv) Next generation sequencing/high throughput sequencing technology has revolutionized genomic and genetic research. The pace of change in this area is significant with, for example, three major new sequencing platforms having been

Table 3. The top 15 species classified at each camp site.

Drill site		Generator site		Kitchen site	
Bradyrhizobium cytisi	6.3%	*Bradyrhizobium cytisi*	21.8%	*Bradyrhizobium cytisi*	18.0%
Arthrobacter psychrochitiniphilus	4.2%	*Brevundimonas staleyi*	17.7%	*Acinetobacter johnsonii*	14.9%
Dokdonella fugitiva	3.2%	*Pelomonas saccharophila*	8.5%	*Enhydrobacter aerosaccus*	11.0%
Bacillus longiquaesitum	3.2%	*Blastococcus aggregatus*	4.0%	*Pelomonas saccharophila*	5.1%
Janthinobacterium lividum	3.2%	*Bradyrhizobium yuanmingense*	3.2%	*Acinetobacter ursingii*	4.5%
Blastococcus aggregatus	3.0%	*Propionibacterium acnes*	3.0%	*Acinetobacter tjernbergiae*	4.2%
Nocardioides islandensis	2.6%	*Micrococcus yunnanensis*	2.3%	*Propionibacterium acnes*	3.3%
Clostridium frigoris	2.0%	*Sphingomonas melonis*	2.1%	*Brevundimonas staleyi*	3.1%
Brevundimonas staleyi	2.0%	*Acidovorax temperans*	1.6%	*Acidovorax temperans*	3.0%
Clostridium vincentii	1.9%	*Pelomonas puraquae*	1.6%	*Bradyrhizobium yuanmingense*	2.9%
Cellulomonas xylanilytica	1.6%	*Pseudonocardia hydrocarbonoxydans*	1.3%	*Pelomonas puraquae*	1.4%
Bacillus nealsonii	1.5%	*Asticcacaulis benevestitus*	1.2%	*Gardnerella vaginalis*	1.1%
Clostridium bowmanii	1.4%	*Enhydrobacter aerosaccus*	1.1%	*Janthinobacterium lividum*	1.1%
Methylobacterium goesingense	1.3%	*Janthinobacterium lividum*	1.1%	*Arthrobacter psychrochitiniphilus*	1.1%
Methylobacterium adhaesivum	1.2%	*Arthrobacter psychrochitiniphilus*	0.9%	*Streptococcus pseudopneumoniae*	0.8%

released in 2011: Ion Torrent's PGM, Pacific Biosciences' RS and the Illumina MiSeq. Quail *et al.* [61] compared the results obtained with the performance of the Illumina HiSeq, the current market leader. There were key differences between the quality of those data reported. (v) The quality and depth of the reference databases—with the race to high throughput sequencing, a lag has been created leaving a credibility gap, whereby the sequence information available is better than the supporting physiology in the sequence databases, i.e. the quality of the data is directly related to the quality of the reference material and not the quality of the sample material collected. This issue will doubtlessly improve, over time, but it limits our present ability to draw functional conclusions about sequence data. This is analogous to the 16S rRNA databases when they were first proposed and started to be constructed. (vi) Repeat sample extraction and analysis do not always generate exactly the same results and many methodologies require some knowledge of the potential targets before they can be applied. Prior to the Lake Ellsworth campaign, we reviewed bacteria taxa that were present in Antarctic supraglacial lakes (table 4). The sample targets were decided based upon the Ellsworth probe's dimensions and transit time, which allowed for the recovery of a total of 21 of lake water and a further 2001 continuous filtration onto the filter membranes. For this reason, the assays available for life detection were constrained by potential sample volume. A polyphasic approach, employing more than one extraction method, is the only practical way around these considerations.

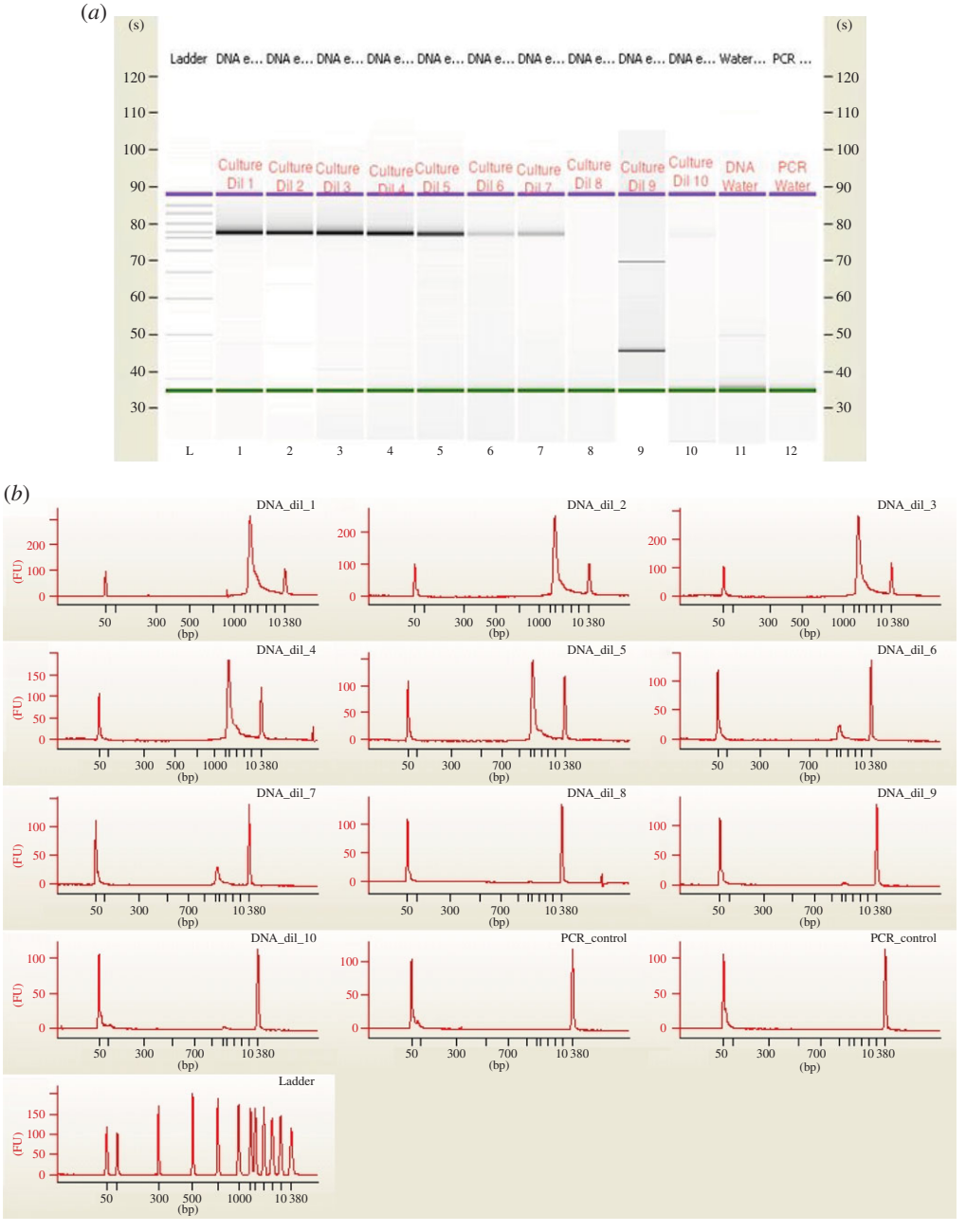

Figure 9. Detection limit assay. (*a*) The detection limit assay showing the amplification generated at different cell concentrations and (*b*) an electrograph of the swab samples and the ladder that was used based on the High Sensitivity DNA kit (Agilent Technologies). (Online version in colour.)

(e) Potential changes to sample integrity

With a significant drop in pressure and a potential for freeze/thaw temperature cycles during sample recovery, the possibility existed of physiological changes inside the sample bottles (figure 13). These significant changes in the environment that the sample is exposed to could lead to cell loss and/or misinterpretation during analysis in a number of ways: (i) freeze–thaw cycling, which can lead to cell lysis; it is also important to know the effect of sample degassing

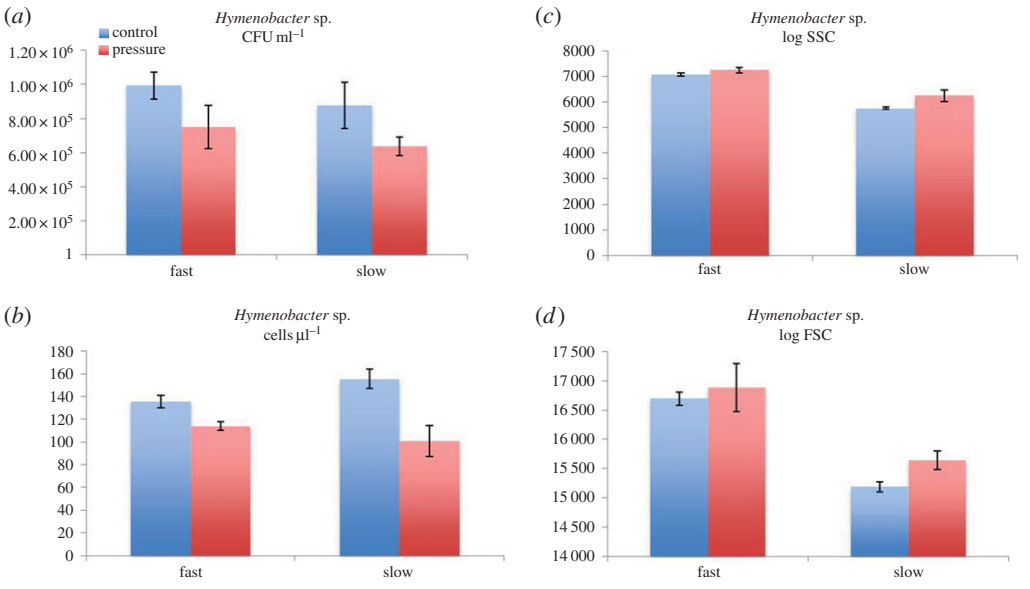

Figure 10. *Hymenobacter* sp. population densities following slow and rapid depressurization. *Hymenobacter* sp. population densities and cytometric characteristic following slow and fast pressurization changes. The figure shows the results as measured by (*a*) direct culture, (*b*) cell abundance, (*c*) log of Side Scattered and (*d*) log of Forward Scattered light.

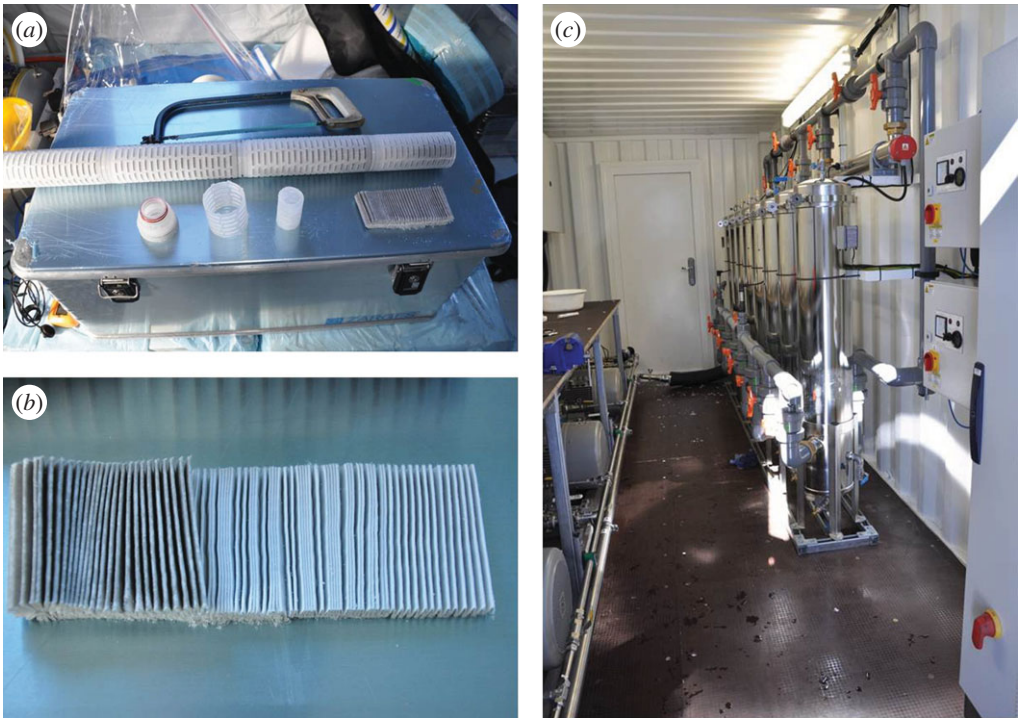

Figure 11. Drill fluid filters. (*a*) Filter units removed, (*b*) filters clogging with carbon particles and (*c*) filter housing *in situ*. (Online version in colour.)

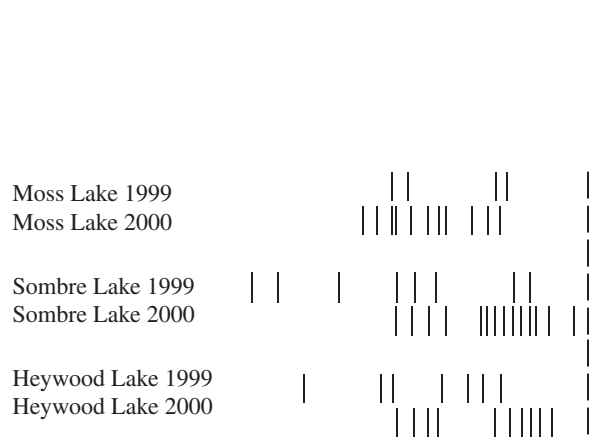

Figure 12. Bacterial community profiles as indicated by denaturing gradient gel electrophoresis profiles from supraglacial Antarctic Lakes across two consecutive field seasons in 1999 and 2000. A transect of trophic status is shown. Moss Lake is oligotrophic (nutrient poor), Sombre Lake is oligotrophic but becoming progressively enriched and Heywood Lake is eutrophic (nutrient rich). Key here is that samples taken in the same way at the same time of year from the same site can yield completely different results.

from the chamber on the microbiological integrity of the sample as although cells can tolerate high or low pressure, the transition from one to another and the speed at which it is done (figure 10); (ii) as sample material is brought back to well-equipped laboratories outside Antarctica, the time that elapses might lead to changes within the sample—it is therefore important to maintain the sample chambers in a closely controlled environment; (iii) it is known that multiple stressors in combination can have a different effect on cells than the combined effects of each stress individually summed together [62], deep Antarctic subglacial lakes are likely to impose many individual stresses [14], and we have yet to generate a full understanding of the implications; and (iv) the issue of cell viability—any RNA work conducted on the sample will be following the time taken to raise the sample from the lake. With a half-life of 30 s in some RNA, this calls into question any interpretation of RNA expression profiles.

Although cells can tolerate high or low pressure, the transition from one to another and the speed at which it is done can lead to cell loss as our preliminary data point out (figure 10*a–d*). In brief, bacteria that have been isolated from the surface snow at the Ellsworth site were pressurized up to 300 bar during a 2 h (slow) and 10 min (fast) period and depressurized to 1 bar within 2 h (slow) and 2 s (fast) using the pressure pot facility at NOCS. Colony forming units (CFU), cell abundance (based on SYBR Green I fluorescence) and scattered light via flow cytometry were measured in pressurized and control (atmospheric pressure) samples. Our preliminary data from the bacteria related to the genus *Hymenobacter* (figure 10*a–d*) show a significant loss in cell viability of approximately 25% (24.6% and 27.6% in fast and slow treatments, respectively) and in cell abundance (15.7% and 35.1% in fast and slow treatments, respectively). Changes in scattered light measured by the Forward (FSC) and Side (SSC) detectors correspond to changes in cell size and membrane integrity and structure. Furthermore, our data indicate that slow pressure change affects more the cell membrane physically compared to the fast pressure change.

Hydrostatic pressure increase is expected to physically disrupt the cell membrane and this disruption can be detected by the intensity of the scattered light detected by the flow cytometer. Based on previous studies [63, and references therein] the intensity of scattered light measured by the Forward detector (FSC) of the flow cytometer corresponds to size changes while the intensity of the scattered light detected by the Side detector (SSC) corresponds to changes in cell membrane integrity and structure. Our data show that *Hymenobacter* cells were significantly affected by pressure changes in the slow rather than fast rate (figure 10*c*,*d*). Those

Table 4. Target genera.

Achromobacter	*Colwellia*	*Mesorhizobium*	*Prochlorococcus*
Acidothermus	*Comamonadaceae*	methanogen	*Propionibacterium*
Acidovorax	*Comamonas*	*Methylobacter*	*Proteiniphilum*
Acinetobacter	*Cyclobacterium*	*Methylobacterium*	*Pseudoalteromonas*
Actinobacterium	*Cytophaga*	*Methylomonas*	*Pseudomonas*
Actinomycetales	*Cytophagales*	*Methylophaga*	*Psychrobacter*
Actinoplanes	*Deinococcus*	*Microbacterium*	*Rhodococcus*
Aeromicrobium	*Desulfosarcina*	*Micrococcus*	*Ralstonia*
Afipia	*Diaphorobacter*	*Microcystis*	*Rhizobium*
Agrobacterium	*Dyadobacter*	*Monosiga*	*Rhizomonas*
Alcaligenes	*Enterococcus*	*Moraxella*	*Rhodobacter*
Alteromonas	*Erythromonas*	*Myxococcus*	*Rhodoferax*
Aquaspirillum	*Flavobacterium*	*Natronohydrobacter*	*Rickettsia*
Arcanobacterium	*Flectobacillus*	*Neisseria*	*Rubritalea*
Arthrobacter	*Flexibacter*	*Nevskia*	*Saligentibacter*
Bacillus	*Frankia*	*Nocardia*	*Shewanella*
Bacteriovorax	*Frigoribacterium*	*Nocardioides*	*Silicibacter*
Bacteroides	*Fusobacterium*	*Nordella*	*Sphingomonas*
Bdellovibrio	*Gelidibacter*	*Nostoc*	*Sphingopyxis*
Beggiatoa	*Geobacter* group	*Nostocoida*	*Spirochaetaceae*
Bordetella	*Glaucimonas*	*Novosphingobium*	*Staphylococcus*
Brachybacteria	*Haliscomenobacter*	*Oceanisphaera*	*Stenotrophomonas*
Bradyrhizobium	*Halobacillus*	*Octadecabacter*	*Sulfitobacter*
Brevundimonas	*Halomonas*	*Oscillatoria*	*Synechococcus*
Burkholderia	*Haslea*	*Oscillatoriales*	*Syntrophus*
Calothrix	*Herbaspirillum*	*Paenibacillus*	*Thauera*
Caulobacter	*Hydrogenophaga*	*Patulibacter*	*Thermus*
Cellulomonas	*Hydrogenophilus*	*Pedobacter*	*Thiobacillus*
Chondrocystis	*Hymenobacter*	*Pelistega*	*Tsukamurella*
Chromobacterium	*Hyphomicrobium*	*Peptostreptococcus*	*Verrumicrobium*
Clavibacter	*Hyphomonas*	*Petalonema*	*Variovorax*
Clostridium	*Imtechium*	*Planktothrix*	*Xanthomonas*
Coccomonas	*Janthinobacterium*	*Planococcus*	
Coleodesmium	*Kineococcus*	*Polaromonas*	
Collimonas	*Marinobacter*	*Polynucleobacter*	138 target genera

data should be handled carefully as they are qualitative rather than quantitative. Further studies incorporating electron microscopy images are needed to define the nature and magnitude of this physical disruption.

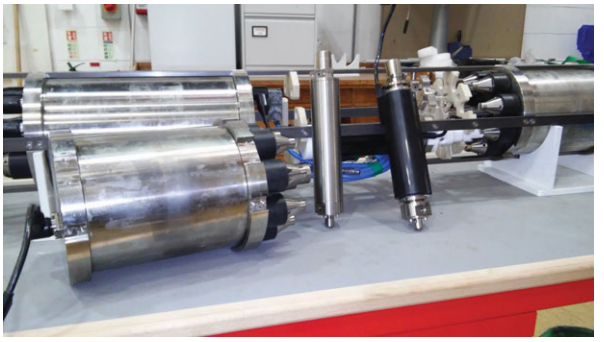

Figure 13. Sample containers used in the probe for recovering water samples. (Online version in colour.)

Table 5. A comparison of two recently studied Antarctic systems.

phylum	Whillans data (%)	Hodgson data (%)
Proteobacteria	75	21
Actinobacteria	1.5	21
Firmicutes	>1	11
Chloroflexi	1	8
Lentisphaerae	>1	<1
Bacteroidetes	10	2
Thaumarchaeota	>1	>1
Planctomycetes	1	16
Verrucomicrobia	1	>1
unclassified	10	20
Proteobacteria by class	**Whillans data (%)**	**Hodgson data (%)**
Alphaproteobacteria	1	11
Betaproteobacteria	60	1
Deltaproteobacteria	5	47
Gammaproteobacteria	6	27
unclassified Proteobacteria	3	14

(f) Recommendations for a future access programme

Our experience in the field during the first attempt to access and sample Lake Ellsworth highlighted a number of important issues relating to the microbiology of deep subglacial lake access.

(i) Context

As one of the sources of potential contamination of samples derived from deep subglacial lakes is the environment of the lake itself, i.e. the supraglacial environment, there is a need to assess each specific local environment when conducting drilling projects, as there will be local differences that it is not possible to predict in advance. Initial results across a range of independent subglacial access projects are starting to suggest that each Antarctic subglacial aquatic ecosystem might be unique (e.g. table 5), hence a single access is unlikely to give us all of the answers.

(ii) Fundamental assumptions

A review of the sterility and cleanliness literature for industry would suggest that more scientifically relevant and informative sterility and cleanliness tests are needed, as we do not yet know whether a single cell is sufficient to colonize a new environment, or indeed, if a threshold cellular concentration is required, what that threshold level is. Indeed, the whole basis of using colony forming units on media needs to be re-examined, as it is of limited scientific relevance.

(iii) Ecological significance

Surprisingly little is still known about microbial colonization processes in natural environments particularly when new species are introduced into pre-existing microbial communities. The factors that lead to successful colonization, growth and reproduction urgently require further study.

(iv) Methodological considerations

Experimentally, bias in specific methodologies such as filtration, DNA extraction, high throughput sequencing, cloning or 16S rRNA gene amplification needs to be accurately determined and assessed for each study undertaken. Detection limits in both experiments for life detection and in the detection of contaminants need to be determined for each field situation and for each experiment. Many of these details are not beyond current technology, and use could be made of analogous environments, mesocosm and microcosm studies, model systems and simulation chambers. To add to this, bacteria from phyla lacking cultivated representatives are widespread in natural systems and some have very small genomes. The limitations of our understanding of colonization processes are important. There is a need of independent verification of the data generated, as microbiology strongly relies on accurate methodology, linked to a deep understanding of which methods are appropriate to test which hypothesis, despite any possible methodological shortcomings; the lack of comparable studies means that any observations made must stand alone until several subglacial systems can be compared.

(v) Future developments

In situ detection methodologies had not been developed in time to be incorporated into the design of the Lake Ellsworth probe. However, the next version of the probe could (and should) incorporate some form of *in situ* biodiversity detection to mitigate the risk of non-sample return. Non-sterility is not an issue in most drilling and subglacial lake access operations, and yet we just do not know the risks yet as we do not yet fully understand the behaviour of microbes. Filtration and verification technologies are not perfect and still need to be improved. However, the potential exists for using melting probes and/or self-sterilization. Currently, we cannot get 100% coverage of biodiversity, though coverage is improving with technological advances. Perhaps most importantly, the critical question of how important microbes are in the functioning of these Antarctic ecosystems still remains.

Authors' contributions. D.A.P.: fieldwork, laboratory work, manuscript, microbiology principal investigator; I.M.: most of the laboratory work and data analysis; G.H.: contributed some of the Mi Seq data (generation and analysis); M.T.: helped collect samples in the field and helped draft the manuscript; M.M.: coordinated sterility testing of the probe, engineering principal investigator; J.W.: project development and helped draft the manuscript; M.J.S.: overall project principal investigator and helped draft the manuscript.

Competing interests. We declare we have no competing interests.

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References

1. Siegert MJ, Priscu JC, Alekhina IA, Wadham JL, Lyons WB. 2016 Antarctic subglacial lake exploration: first results and future plans. *Phil. Trans. R. Soc. A* **374**, 20140466. (doi:10.1098/rsta.2014.0466)
2. Wright A, Siegert M. 2012 A fourth inventory of Antarctic subglacial lakes. *Antarct. Sci.* **24**, 659–664. (doi:10.1017/S095410201200048X)
3. Siegert MJ, Ross N, Le Brocq AM. 2016 Recent advances in understanding Antarctic subglacial lakes and hydrology. *Phil. Trans. R. Soc. A* **374**, 20140306. (doi:10.1098/rsta.2014.0306)
4. Siegert M *et al.* 2007 Exploration of Ellsworth Subglacial Lake: a concept paper on the development, organisation and execution of an experiment to explore, measure and sample the environment of a West Antarctic subglacial lake. *Rev. Environ. Sci. Biotechnol.* **6**, 161–179. (doi:10.1007/s11157-006-9109-9)
5. Priscu JC *et al.* 2003 An international plan for Antarctic subglacial lake exploration. *Polar Geogr.* **27**, 69–83. (doi:10.1080/789610223)
6. Christner BC *et al.* 2014 A microbial ecosystem beneath the West Antarctic ice sheet. *Nature* **512**, 310–313. (doi:10.1038/nature13667)
7. Ellis-Evans JC, Wynn-Williams D. 1996 Antarctica: a great lake under the ice. *Nature* **381**, 644–646. (doi:10.1038/381644a0)
8. Inagaki F *et al.* 2015 Exploring deep microbial life in coal-bearing sediment down to ~2.5 km below the ocean floor. *Science* **349**, 420–424. (doi:10.1126/science.aaa6882)
9. Lanoil B, Skidmore M, Priscu JC, Han S, Foo W, Vogel SW, Tulaczyk S, Engelhardt H. 2009 Bacteria beneath the West Antarctic ice sheet. *Environ. Microbiol.* **11**, 609–615. (doi:10.1111/j.1462-2920.2008.01831.x)
10. Christner BC *et al.* 2006 Limnological conditions in subglacial Lake Vostok, Antarctica. *Limnol. Oceanogr.* **51**, 2485–2501. (doi:10.4319/lo.2006.51.6.2485)
11. Mikucki JA, Priscu JC. 2007 Bacterial diversity associated with Blood Falls, a subglacial outflow from the Taylor Glacier, Antarctica. *Appl. Environ. Microbiol.* **73**, 4029–4039. (doi:10.1128/AEM.01396-06)
12. Murray AE *et al.* 2012 Microbial life at −13°C in the brine of an ice-sealed Antarctic lake. *Proc. Natl Acad. Sci. USA* **109**, 20 626–20 631. (doi:10.1073/pnas.1208607109)
13. Pearce DA, Hodgson DA, Thorne MA, Burns G, Cockell CS. 2013 Preliminary analysis of life within a former subglacial lake sediment in Antarctica. *Diversity* **5**, 680–702. (doi:10.3390/d5030680)
14. Siegert MJ *et al.* 2012 Clean access, measurement, and sampling of Ellsworth Subglacial Lake: a method for exploring deep Antarctic subglacial lake environments. *Rev. Geophys.* **50**, RG1003. (doi:10.1029/2011RG000361)
15. Siegert MJ, Makinson K, Blake D, Mowlem M, Ross N. 2014 An assessment of deep-hot-water drilling as a means to undertake direct measurement and sampling of Antarctic subglacial lakes: experience and lessons learned from the Lake Ellsworth. *Ann. Glaciol.* **55**, 59–73. (doi:10.3189/2014AoG65A008)
16. Woodward J *et al.* 2010 Location for direct access to subglacial Lake Ellsworth: an assessment of geophysical data and modeling. *Geophys. Res. Lett.* **37**, L11501. (doi:10.1029/2010GL042884)
17. Pearce DA, Bridge PD, Hughes KA, Sattler B, Psenner R, Russell NJ. 2009 Microorganisms in the atmosphere over Antarctica. *FEMS Microbiol. Ecol.* **69**, 143–157. (doi:10.1111/j.1574-6941.2009.00706.x)
18. Lopatina A, Krylenkov V, Severinov K. 2013 Activity and bacterial diversity of snow around Russian Antarctic stations. *Res. Microbiol.* **164**, 949–958. (doi:10.1016/j.resmic.2013.08.005)
19. Segawa T *et al*. 2010 Bacterial communities in two Antarctic ice cores analyzed by 16S rRNA gene sequencing analysis. *Polar Sci.* **4**, 215–227. (doi:10.1016/j.polar.2010.05.003)
20. Lanoil B *et al*. 2009 Bacteria beneath the West Antarctic ice sheet. *Environ. Microbiol.* **11**, 609–615. (doi:10.1111/j.1462-2920.2008.01831.x)

21. Shtarkman YM *et al*. 2013 Subglacial Lake Vostok (Antarctica) accretion ice contains a diverse set of sequences from aquatic, marine and sediment-inhabiting bacteria and eukarya. *PLoS ONE* **8**, e67221. (doi:10.1371/journal.pone.0067221)
22. Rogers SO *et al*. 2013 Ecology of subglacial Lake Vostok (Antarctica), based on metagenomic/metatranscriptomic analyses of accretion ice. *Biology* **2**, 629–650. (doi:10.3390/biology2020629)
23. Mikucki JA, Priscu JC. 2007 Bacterial diversity associated with Blood Falls, a subglacial outflow from the Taylor Glacier, Antarctica. *Appl. Environ. Microbiol.* **73**, 4029–4039. (doi:10.1128/AEM.01396-06)
24. Van Houdt R *et al*. 2013 Characterization of culturable *Paenibacillus* spp. from the snow surface on the high Antarctic Plateau (Dome C) and their dissemination in the Concordia research station. *Extremophiles* **17**, 565–573. (doi:10.1007/s00792-013-0539-3)
25. Antony R *et al*. 2012 Diversity and physiology of culturable bacteria associated with a coastal Antarctic ice core. *Microbiol. Res.* **167**, 372–380. (doi:10.1016/j.micres.2012.03.003)
26. Alekhina IA *et al*. 2007 Molecular analysis of bacterial diversity in kerosene-based drilling fluid from the deep ice borehole at Vostok, East Antarctica. *FEMS Microbiol. EcoL.* **59**, 289–299. (doi:10.1111/j.1574-6941.2006.00271.x)
27. Christner BC *et al*. 2001 Isolation of bacteria and 16S rDNAs from Lake Vostok accretion ice. *Environ. Microbiol.* **3**, 570–577. (doi:10.1046/j.1462-2920.2001.00226.x)
28. Fujii M *et al*. 2010 Microbial community structure, pigment composition, and nitrogen source of red snow in Antarctica. *Microb. EcoL.* **59**, 466–475. (doi:10.1007/s00248-009-9594-9)
29. García-Echauri S *et al*. 2011 Isolation and phylogenetic classification of culturable psychrophilic prokaryotes from the Collins Glacier in Antarctica. *Folia Microbiol.* **56**, 209–214. (doi:10.1007/s12223-011-0038-9)
30. Gonzalez-Toril E *et al*. 2009 Bacterial diversity of autotrophic enriched cultures from remote, glacial Antarctic, Alpine and Andean aerosol, snow and soil samples. *Biogeosciences* **6**, 33–44. (doi:10.5194/bg-6-33-2009)
31. Priscu JC *et al*. 1999 Geomicrobiology of subglacial ice above Lake Vostok, Antarctica. *Science* **286**, 2141–2144. (doi:10.1126/science.286.5447.2141)
32. Klassen JL, Foght JM. 2011 Characterization of *Hymenobacter* isolates from Victoria Upper Glacier, Antarctica reveals five new species and substantial non-vertical evolution within this genus. *Extremophiles* **15**, 45–57. (doi:10.1007/s00792-010-0336-1)
33. Carpenter EJ, Lin S, Capone DG. 2000 Bacterial activity in South Pole snow. *Appi. Environ. Microbiol.* **66**, 4514–4517. (doi:10.1128/AEM.66.10.4514-4517.2000)
34. Pearce DA, Newsham KK, Thorne MA, Calvo-Bado L, Krsek M, Laskaris P, Hodson A, Wellington EM. 2012 Metagenomic analysis of a southern maritime Antarctic soil. *Front. Microbiol.* **3**, 403. (doi:10.3389/fmicb.2012.00403)
35. Lazzaro A, Wismer A, Schneebeli M, Erny I, Zeyer J. 2015 Microbial abundance and community structure in a melting alpine snowpack. *Extremophiles* **19**, 631–642. (doi:10.1007/s00792-015-0744-3)
36. Zhang S, Hou S, Qin X, Du W, Liang F, Li Z. 2015 Preliminary study on effects of glacial retreat on the dominant glacial snow bacteria in Laohugou glacier no. 12. *Geomicrobiol. J.* **32**, 113–118. (doi:10.1080/01490451.2014.929761)
37. Larose C, Dommergue A, Vogel TM. 2013 The dynamic arctic snow pack: an unexplored environment for microbial diversity and activity. *Biology* **2**, 317–330. (doi:10.3390/biology2010317)
38. Larose C, Dommergue A, Vogel TM. 2013 Microbial nitrogen cycling in Arctic snowpacks. *Environ. Res. Lett.* **8**, 035004. (doi:10.1088/1748-9326/8/3/035004)
39. Cameron KA, Hagedorn B, Dieser M, Christner BC, Choquette K, Sletten R, Crump B, Kellogg C, Junge K. 2015 Diversity and potential sources of microbiota associated with snow on western portions of the Greenland Ice Sheet. *Environ. Microbiol.* **17**, 594–609. (doi:10.1111/1462-2920.12446)
40. Choudhari S, Lohia R, Grigoriev A. 2014 Comparative metagenome analysis of an Alaskan glacier. *J. Bioinform. Comput. Biol.* **12**, 1441003. (doi:10.1142/S0219720014410030)
41. Carpenter EJ, Lin S, Capone DG. 2000 Bacterial activity in South Pole snow. *Appl. Environ. Microbiol.* **66**, 4514–4517. (doi:10.1128/AEM.66.10.4514-4517.2000)

42. Skidmore ML, Foght JM, Sharp MJ. 2000 Microbial life beneath a high Arctic glacier. *Appl. Environ. Microbiol.* **66**, 3214–3220. (doi:10.1128/AEM.66.8.3214-3220.2000)
43. Pearce DA. 2005 The structure and stability of the bacterioplankton community in Antarctic freshwater lakes, subject to extremely rapid environmental change. *FEMS Microbiol. Ecol.* **53**, 61–72. (doi:10.1016/j.femsec.2005.01.002)
44. Frick A, Mogul R, Stabekis P, Conley CA, Ehrenfreund P. 2014 Overview of current capabilities and research and technology developments for planetary protection. *Adv. Space Res.* **54**, 221–240. (doi: 10.1016/j.asr.2014.02.016)
45. Green V. 1993 Surgery, sterilization and sterility. *J. Healthcare Mater. Manage.* **46**, 48–52.
46. Chattopadhyay M. 2000 Cold-adaptation of Antarctic microorganisms—possible involvement of viable but nonculturable state. *Polar Biol.* **23**, 223–224. (doi:10.1007/s003000050030)
47. Mowlem M *et al.* 2016 Probe technologies for clean sampling and measurement of subglacial lakes. *Phil. Trans. R. Soc. A* **374**, 20150267. (doi:10.1098/rsta.2015.0267)
48. Kwan K *et al.* 2011 Evaluation of procedures for the collection, processing, and analysis of biomolecules from low-biomass surfaces. *Appl. Environ. Microbiol.* **77**, 2943–2953. (doi:10.1128/Aem.02978-10)
49. Caporaso JG *et al.* 2011 Global patterns of 16S rRNA diversity at a depth of millions of sequences per sample. *Proc. Natl Acad. Sci. USA* **108**, 4516–4522. (doi:10.1073/pnas.1000080107)
50. King EO, Ward MK, Raney DE. 1954 Two simple media for the demonstration of pyocyanin and fluorescein. *J. Lab. Clin. Med.* **44**, 301–307.
51. Reasoner DJ, Geldreich EE. 1985 A new medium for the enumeration and subculture of bacteria from potable water. *Appl. Environ. Microbiol.* **49**, 1–7.
52. Altschul SF, Gish W, Miller W, Myers EW, Lipman DJ. 1990 Basic local alignment search tool. *J. Mol. Biol.* **215**, 403–410. (doi:10.1006/jmbi.1990.9999)
53. Siegert MJ, Ellis-Evans JC, Tranter M, Mayer C, Petit JR, Salamatin A, Priscu JC. 2001 Physical, chemical and biological processes in Lake Vostok and other Antarctic subglacial lakes. *Nature* **414**, 603–609. (doi:10.1038/414603a)
54. Shtarkman YM, Koçer ZA, Edgar R, Veerapaneni RS, D'Elia T, Morris PF, Rogers SO. 2013 Subglacial Lake Vostok (Antarctica) accretion ice contains a diverse set of sequences from aquatic, marine and sediment-inhabiting bacteria and eukarya. *PLoS ONE* **8**, e67221. (doi:10.1371/journal.pone.0067221)
55. Venkateswaran K, Kempf M, Chen F, Satomi M, Nicholson W, Kern R. 2003 *Bacillus nealsonii* sp. nov., isolated from a spacecraft-assembly facility, whose spores are γ-radiation resistant. *Int. J. Syst. Evol. Microbiol.* **53**, 165–172. (doi:10.1099/ijs.0.02311-0)
56. Wang F, Gai Y, Chen M, Xiao X. 2009 *Arthrobacter psychrochitiniphilus* sp. nov., a psychrotrophic bacterium isolated from Antarctica. *Int. J. Syst. Evolut. Microbiol.* **59**, 2759–2762. (doi:10.1099/ijs.0.008912-0)
57. Segawa T, Miyamoto K, Ushida K, Agata K, Okada N, Kohshima S. 2005 Seasonal change in bacterial flora and biomass in mountain snow from the Tateyama Mountains, Japan, analyzed by 16S rRNA gene sequencing and real-time PCR. *Appl. Environ. Microbiol.* **71**, 123–130. (doi:10.1128/AEM.71.1.123-130.2005)
58. Luef B *et al.* 2015 Diverse uncultivated ultra-small bacterial cells in groundwater. *Nature Commun.* **6**, 6372. (doi:10.1038/ncomms7372)
59. Vishnivetskaya TA *et al.* 2014 Commercial DNA extraction kits impact observed microbial community composition in permafrost samples. *FEMS Microbiol. Ecol.* **87**, 217–230. (doi:10.1111/1574-6941.12219)
60. Lynch MD, Neufeld JD. 2015 Ecology and exploration of the rare biosphere. *Nat. Rev. Microbiol.* **13**, 217–229. (doi:10.1038/nrmicro3400)
61. Quail MA, Smith M, Coupland P, Otto TD, Harris SR, Connor TR, Bertoni A, Swerdlow HP, Gu Y. 2012 A tale of three next generation sequencing platforms: comparison of Ion Torrent, Pacific Biosciences and Illumina MiSeq sequencers. *BMC Genomics* **13**, 341. (doi:10.1186/1471-2164-13-341)
62. Harrison JP, Gheeraert N, Tsigelnitskiy D, Cockell CS. 2013 The limits for life under multiple extremes. *Trends Microbiol.* **21**, 204–212. (doi:10.1016/j.tim.2013.01.006)
63. Shapiro HM. 2003 *Practical flow cytometry*, 4th edn. Hoboken, NJ: John Wiley.